

HUMANIZED ANTIBODY AND PROCESS FOR PREPARING SAME

Field of the Invention

5 The present invention relates to a process for preparing a humanized antibody by grafting SDRs (specificity determining residues) in CDRs (complementary determining residues) of murine monoclonal antibody to human antibody and the humanized antibody prepared according to said process.

10

Background of the Invention

15 For preventing infectious diseases such as hepatitis B, there has generally been used a method of administering immunoglobulins formed in blood plasma against a target antigen. However, the method has the problems that the immunoglobulins generally have low specificity and may contain contaminants.

20 Murine monoclonal antibody derived from mouse has been reported to have high affinity to antigen and is suitable for mass-production. However, repeated injection of murine monoclonal antibody induces an immune response because the murine antibody is regarded as a foreign antigen in humans (Shawler D.L. et al., *J. Immunol.*, 135, 1530-1535(1985)).

25 Accordingly, numerous efforts have been made to generate "humanized antibody" by: grafting the CDR (complementarity determining region) of murine monoclonal antibody variable region which directly binds to antigens, to a human antibody framework (CDR-grafting method); and replacing the amino acid residues of the human antibody framework region (FR) that influence the CDR conformation with the amino acid residues of murine monoclonal antibody. The humanized antibody thus obtained maintains the affinity and specificity of original murine monoclonal antibody, and minimizes HAMA(human anti-mouse antibody) response in humans (Riechmann et al., *Nature*, 332, 323-327(1988); Queen C. et al., *Proc. Natl. Acad. Sci. USA*, 86, 10029-10033(1989); Nakatani et al., *Protein Engineering*, 7, 435-443(1994)). However, this humanized antibody still causes problems when injected repeatedly into humans (Stephens et al., 30 *Immunology*, 85, 668-674(1995); Sharkey et al., *Cancer Research*, 55, 5935s-5945s(1995)).

Approximately 300 millions of world population carry hepatitis B virus ("HBV") which may cause chronic infection, leading to cirrhosis and hepatocellular carcinoma (Tiollais P. and Buendia M.A., *Sci. Am.*, 264, 48(1991)). The HBV envelope consists of three proteins, major protein containing S antigen, middle protein containing S and pre-S2 antigens, and large protein containing S, pre-S2 and pre-S1 antigens (Neurath A.R. and Kent S.B., *Adv. Vir. Res.*, 34, 65-142(1988)). These surface antigens have been known to play important roles in the process of forming antibodies against HBV in hepatitis patient. The pre-S1 region, in particular, is found on infectious viral particles (Heermann et al., *J. Virol.*, 52, 396-402(1984)) and plays a role in attachment to cell surface infection (Neurath et al., *Cell*, 46, 429(1986); Pontisso et al., *Virol.*, 173, 533, (1989); Neurath et al., *Vaccine*, 7, 234(1989)). Thus a monoclonal antibody against the pre-S1 would be effective against viral infection.

The present inventors have previously reported a murine monoclonal antibody (KR127) against HBV pre-S1 (Korean Patent No. 246128), a murine monoclonal antibody KR127 gene encoding same (Korean Patent No. 250832) and a humanized antibody (HZKP127I) of KR127 prepared by CDR-grafting method (Korean Patent No. 246128).

The present inventors have further endeavored to develop a humanized antibody having minimized adverse immune response (HAMA response) as well as enhanced affinity to antigen, and found that HAMA response can be reduced when the amino acid residues of CDR of mouse antibody are replaced with those of human antibody.

Summary of the Invention

Accordingly, it is an object of the present invention to provide a process for preparing a humanized antibody which is expected to show lower HAMA response and has higher affinity than humanized antibody of the prior art.

It is another object of the present invention to provide a humanized antibody prepared according to said process.

It is a further another object of the present invention to provide a DNA encoding the heavy chain or light chain of said antibody and a vector comprising said DNA.

It is a still further object of the present invention to provide a

microorganism transformed with said vector.

In accordance with one aspect of the present invention, there is provided a process for preparing a humanized antibody comprising the steps of: (a) selecting a specificity determining residue (SDR) of the complementarity determining region (CDR) of murine monoclonal antibody heavy chain and light chain variable regions; and (b) grafting the amino acid residues of said SDR to at least one of the corresponding amino acid sequences in human antibody variable regions.

10 Brief Description of the Drawings

The above and other objects and features of the present invention will become apparent from the following description of the invention taken in conjunction with the following accompanying drawings; which 15 respectively show:

Fig. 1 : the procedure for constructing an expression vector of a chimeric heavy chain;

Fig. 2 : the nucleotide and amino acid sequence of the humanized 20 heavy chain variable region;

Fig. 3 : the procedure for constructing an expression vector of a chimeric light chain;

Fig. 4 : the nucleotide and amino acid sequence of the humanized light chain variable region;

25 Fig. 5 : the affinity to antigen of a humanized antibody having a heavy chain CDR mutant;

Fig. 6 : the procedure for constructing an expression vector of the humanized antibody; and

30 Figs. 7 and 8 : the results of analysis for MHC class II-binding peptide sequences in heavy chain variable regions of HuKR127 and light chain variable regions of HuKR127, respectively, which are compared with HzKR127I, respectively.

Detailed Description of the Invention

35

The humanized antibody of the present invention may be prepared by

grafting the amino acid residues of SDR of murine monoclonal antibody to the corresponding amino acid sequences in human antibody variable regions.

SDRs of the murine monoclonal antibody used in the present invention may be determined by independently replacing each amino acid residue of CDR of the murine monoclonal antibody with alanine, selecting 5 transformants which have lower affinity (k_D) to antigen than the original murine antibody and determining the replaced CDR amino acid residues of said transformants as SDRs.

Further, in order to enhance the affinity to antigen, the CDR residues 10 of a mouse antibody that increase the affinity and the framework residues that influence the conformation of CDR loops may also be grafted to the corresponding sites of human antibody.

For example, the present invention describes a process for preparing 15 a humanized antibody for hepatitis B virus (HBV) pre-S1 by using murine monoclonal antibody KR127 (Korean Patent No. 250832) as follows:

After selecting SDR amino acid residues, which play important roles 20 in binding with antigen, from CDR of the murine monoclonal antibody KR127 heavy and light chains, chimeric heavy chain and chimeric light chain genes may be prepared by combining either the variable region of KR127 antibody heavy chain with the constant region ($C_{\gamma} 1$) of human antibody or the variable region of KR127 antibody light chain with the constant region (C_{κ}) of human antibody.

SDRs of the murine monoclonal antibody for HBV pre-S1 are 25 determined by replacing each amino acid residue of CDR HCDR1 (aa 31-35), HCDR2 (aa 50-65) and HCDR3 (aa 95-102) of the heavy chain (SEQ ID NO: 2) and CDR LCDR1 (aa 24-34), LCDR2(aa 50-56) and LCDR3(aa 89-97) of the light chain (SEQ ID NO: 4) of the murine monoclonal antibody KR127 with alanine according to the alanine scanning mutagenesis method 30 and selecting the amino acid residues (SDRs) whose replacement with alanine bring about more than 3 times reduction in the affinity to antigen(K_D) as compared with the original murine antibody. Throughout this description, amino acid residue number is assigned according to Kabat numbering scheme (Kabat, E. A. et al, Sequences of Proteins of Immunological Interest. *National Institute of Health, Bethesda, MD.*, 1991).

35 Examples of preferred SDR include tryptophan at position 33 (it is represented as "Trp33"), Met34, and Asn35 of HCDR1; Arg50, Tyr52, and

Pro52a of HCDR2; Glu95, Tyr96, and Glu98 of HCDR3 of the murine monoclonal antibody KR127 heavy chain; Leu27b, Tyr27d, Ser27e; Asn28, Lys30, Tyr32, and Asn34 of LCDR1; Leu50 and Asp55 of LCDR2; and Val89, Gln90, Gly91, Thr92, His93, Phe94, Pro95, and Gln96 of LCDR3 of the murine monoclonal antibody KR127 light chain.

The humanized antibody of the present invention can be prepared by grafting one or more SDRs determined as above onto the human antibody heavy chain and/or light chain. The human antibody heavy chain which may be used in the present invention is human heavy chain DP7-JH4 consisting of human immunoglobulin germline VH gene segment DP7 (Tomlinson et al., *J. Mol. Biol.*, 227, 776-798, 1992) and JH4 segment (Ravetch et al., *Cell*, 27, 583-591, 1981). The human antibody light chain which may be used in the present invention is human light chain DPK12-JH4 consisting of human immunoglobulin germline VK gene segment DPK12 (Cox et al., *Eur. J. Immunol.*, 24, 827-836 (1994)) and JH4 segment (Hieter et al., *J. Biol. Chem.*, 257, 1516-1522 (1982)).

The humanized heavy chain of the present invention may be prepared by grafting at least one of Trp33, Met34, and Asn35 of HCDR1; Arg50, Tyr52, and Pro52a of HCDR2; Glu95, Tyr96, and Glu98 of HCDR3 of the murine monoclonal antibody KR127 heavy chain to the corresponding amino acid sequences in human antibody heavy chain. The inventive humanized light chain may be prepared by grafting at least one of Leu27b, Tyr27d, Ser27e; Asn28, Lys30, Tyr32, and Asn34 of LCDR1; Leu50 and Asp55 of LCDR2; and Val89, Gln90, Gly91, Thr92, His93, Phe94, Pro95, and Gln96 of LCDR3 of the murine monoclonal antibody KR127 light chain to the corresponding amino acid sequences in human antibody light chain DPH12-JK4.

Moreover, the affinity to antigen of the humanized antibody can be enhanced by the follow substitutions:

- 30 (a) the amino acid residue at position 32 in HCDR1 of the modified human heavy chain DP7-JH4 by Ala;
- 35 (b) the amino acid residue at position 97 in HCDR3 of the modified human heavy chain DP7-JH4 by Arg or Ala;
- (c) the amino acid residue at position 98 in HCDR3 of the modified human heavy chain DP7-JH4 by Val; and
- (d) the amino acid residue at position 102 in HCDR3 of the

modified human heavy chain DP7-JH4 by Arg or Ala.

In addition, Ala71 and Lys73 in framework region 3 in the heavy chain variable region of KR127, which affects the conformation of the CDR loop, may further be grafted to human heavy chain DP7-JH4. Also, Leu36 and Arg46 in framework region 2 in the light chain variable region of KR127, which affects conformation of CDR loop, may be further grafted to human light chain DPH12-JK4.

The heavy chain variable region of humanized antibody of the present invention has the amino acid sequence of SEQ ID NO: 2, preferably encoded by the nucleotide sequence of SEQ ID NO: 1 and the inventive light chain variable region of humanized antibody has the amino acid sequence of SEQ ID NO: 4, preferably encoded by the nucleotide sequence of SEQ ID NO: 3.

The humanized antibody heavy chain and light chain of the present invention may be encoded by a gene comprising a nucleotide sequence deduced from the humanized antibody heavy chain and light chain according to the genetic code. It is known that several different codons encoding a specific amino acid may exist due to the codon degeneracy, and, therefore, the present invention includes in its scope all nucleotide sequences deduced from the humanized antibody heavy chain and light chain amino acid sequence. Preferably, the humanized antibody heavy chain and light chain gene sequences include one or more preferred codons of host cell.

The humanized antibody consisted of the humanized heavy chain HuKR127HC of the present invention and humanized light chain HZKR127I prepared by CDR-grafting has an affinity to antigen of about over 50 times higher than that of the humanized antibody HZKR127I.

The humanized antibody consisting of the humanized heavy chain HuKR127KC of the present invention and humanized light chain HZKR127I prepared by CDR-grafting has an affinity to antigen equal to that of the humanized antibody HZKR127I.

The genes of humanized antibody heavy chain and light chain thus prepared may be inserted to pdCMV-dhfrC-HAV6 vector (KCTC 10028BP) to obtain an expression vector pdCMV-dhfrC-HuKR127 which can express both humanized antibody heavy chain HuKR127HC and light chain HZKR127I. The expression vector of the present invention may be introduced into microorganism, e.g., *E. coli* DH5 α according to a

conventional transformation method to obtain transformants *E. coli* DH5a / pdCMV-dhfrC-HuKR127. The transformants *E. coli* DH5a / pdCMV-dhfrC-HuKR127 was deposited on March 13, 2002 with the Korean Collection for Type Cultures(KCTC)(Address: Korea Research Institute of Bioscience and Biotechnology(KRIBB), #52, Oun-dong, Yusong-ku, Taejon, 305-333, Republic of Korea) under the accession number, KCTC 10198BP, in accordance with the terms of Budapest Treaty on the International Recognition of the Deposit of Microorganism for the Purpose of Patent Procedure.

Meanwhile, CHO/HuKR127, CHO (Chinese hamster ovary) cell line transfected with vector pdCMV-dhfrC-HuKR127, was deposited on March 13, 2002 with the Korean Collection for Type Cultures(KCTC) under the accession number, KCTC 10199BP, in accordance with the terms of Budapest Treaty on the International Recognition of the Deposit of Microorganism for the Purpose of Patent Procedure.

The humanized antibody HuKR127 of the present invention produced by culturing the CHO/HuKR127 cell line has a higher affinity to antigen and is expected to reduce HAMA (human anti-mouse antibody) response to a greater extent than the conventional antibody prepared according to the CDR-grafting method.

Accordingly, the humanized antibody of the present invention can be used in preventing hepatitis B virus infection and treating chronic Hepatitis B.

Thus, for preventing hepatitis B virus infection and treating chronic Hepatitis B, a pharmaceutical formulation of the inventive humanized antibody may be prepared in accordance with any of the conventional procedures.

The pharmaceutical composition of the present invention can be administered via various routes including intravenous and intramuscular introduction. It should be understood that the amount of the active ingredient actually administered ought to be determined in light of various relevant factors including the condition to be treated, the chosen route of administration, the age, sex and body weight of the individual patient, and the severity of the patient's symptom; and, therefore, the above dose should not be intended to limit the scope of the invention in any way.

The following Examples are intended to further illustrate the present invention without limiting its scope.

Example 1: Preparation of mouse/human chimeric heavy chain gene

5 The gene encoding leader sequence and the $\gamma 1$ constant region of the human antibody heavy chain were separately prepared by carrying out PCR using pCMV-HKR127HC (Korean Patent No. 246128, KCTC 0531BP) as a template and a primer set of Ryu94 (SEQ ID NO: 5) and HUR43-1 (SEQ ID NO: 6) or HUR46-1 (SEQ ID NO: 9) and HUR31(SEQ ID NO: 10).

10 The gene encoding heavy chain variable region of the murine monoclonal antibody KR127 was prepared by carrying out PCR using pKR127H(Korean Patent No. 250832, KCTC 0333BP) as a template and primers HUR44-1(SEQ ID NO: 7) and HUR45-1(SEQ ID NO: 8).

15 Ryu94: 5'-GAG AAT TCA CAT TCA CGA TGT ACT TG-3'
HUR43-1: 5'-CTG CTG CAG CTG GAC CTG ACT CTG GAC ACC ATT-
3'
HUR44-1: 5'-CAG GTC CAG CTG CAG CAG TCT GGA CCT GAA CTG-
3'
20 HUR45-1: 5'-TGG GCC CTT GGT GGA GGC TGC AGA GAC AGTGAC-
3'
HUR46-1: 5'-GCC TCC ACC AAG GGC CCA TCG GTC TTC CCC CTG-
3'
HUR31: 5'-CAG CGG CCG CTC ATT TAC CCG GGG ACA G-3'

25 Each PCR reaction was carried out using 10 ng of template, 1 μ l of each primer (50 ppm), 0.5 μ l of *Pfu* DNA polymerase (Promega), 4 μ l of 2.5 mM dNTPmix and 5 μ l of 10 x *Pfu* reaction buffer solution. After pre-denaturation at 95 °C for 5 minutes, a PCR cycle was repeated 25 times, the cycle being composed of: 95 °C for 30 sec., 50 °C for 30 sec. and 72 °C for 45 sec. After annealing the DNA fragment obtained by using primers Ryu94 and HUR43-1, the DNA fragment obtained by using primers HUR44-1 and HUR45-1, and the DNA fragment obtained by using primers HUR46-1 and HUR31 were recombined by conducting recombinant PCR using primers Ryu94 and HUR31. The recombinant PCR reaction was carried out using the same reaction buffer solution as used above. After pre-denaturation

at 95 °C for 5 minutes, a PCR cycle was repeated 30 times, the cycle being composed of: 95 °C for 30 sec., 50 °C for 30 sec. and 72 °C for 60 sec., and finally, the extension reaction was carried out at 72 °C for 5 min.

5 The chimeric heavy chain gene thus prepared was cleaved with *Eco*RI(GAATTC) and *Nde*I (GCGGCCGC) and inserted at the *Eco*RI/*Nde*I section of vector pcDdA (plasmid which is removed ApaI site in the multiple cloning site of pcDNA received from Invitrogen), to obtain vector pcDdAchKR127HC (Fig. 1). The base sequence of the chimeric heavy chain variable region gene (KR127VH) was confirmed by DNA sequence 10 analysis (Fig. 2).

Example 2: Preparation of mouse/human chimeric light chain gene

15 The gene encoding reader sequence and the constant region of the human antibody light chain were each prepared by carrying out PCR using pKC-dhfr-HKR127 (Korean Patent No. 2000-33008, KCTC 0529BP) as a template and a primer set of Ryu86 (SEQ ID NO: 11) and HUR48 (SEQ ID NO: 12) or HUR51 (SEQ ID NO: 15) and CK1D (SEQ ID NO: 16).

20 The gene encoding light chain variable region of the murine monoclonal antibody KR127 was prepared by carrying out PCR using pKR127K (Korean Patent No. 250832, KCTC 0334BP) as a template and primers HUR49 (SEQ ID NO: 13) and HUR50 (SEQ ID NO: 14).

Ryu86: 5'-CAA AGC TTG GAA GCA AGA TGG ATT CA-3'

25 HUR48: 5'-CAA GAT ATC CCC ACA GGT ACC AGA TAC-3'

HUR49: 5'-TGT GGG GAT ATC TTG ATG ACC CAA ACT-3'

HUR50: 5'-CAC AGA TCT TTT GAT TTC CAG CTT GGT-3'

HUR51: 5'-ATC AAA AGA TCT GTG GCT GCA CCA TCT-3'

CK1D: 5'-GCG CCG TCT AGA ATT AAC ACT CTC CCC TGT TGA 30 AGC TCT TTG TGA CGG GCG AACTCAG-3'

Each PCR reaction was carried out according to the method described in Example 1 except that primers Ryu86 and CK1D were used to ligate the annealed DNA fragments obtained by PCR reactions.

35 The chimeric light chain gene thus prepared was cleaved with *Hind*III (AAGCTT) and *Xba*I (TCTAGA) and inserted at the *Hind*III/*Xba*I

section of vector pBluescript KS, to obtain a recombinant plasmid. Subsequently, the recombinant plasmid was cleaved with *Hind*III and *Apal* and inserted at the *Hind*III/*Apal* section of vector pCMV-dhfr (KCTC 8671P), to obtain plasmid pKC-dhfr-chKR127(Fig. 3). The base sequence 5 of the chimeric light chain variable region gene (KR127VK) was confirmed by DNA sequence analysis (Fig. 4).

Example 3: Mutation of CDR of chimeric KR127 antibody heavy chain by alanine injection

10 To examine whether each amino acid residue of KR127 heavy chain HCDR1 (aa 31-35), HCDR2(aa 50-65) and HCDR3 (aa 95-102) binds to antigen, PCR reaction was carried out using vector pcDdA-chKR127HC as a template to prepare a modified gene, wherein an amino acid residue of CDR 15 was replaced with alanine (the replaced amino acid residue No. was indicated as Kabat number) (see Fig. 2).

20 A forward primer YM001N of SEQ ID NO: 17 was designed to provide the sequence corresponding to the reader sequence at the 5'-end of the chimeric heavy chain gene and *Eco*RI restriction site, and a reverse primer YM003 of SEQ ID NO: 18 was designed to have the sequence 25 corresponding to the N-terminal downstream of CH1 domain of human heavy chain gene and *Apal* restriction site.

YM001N: 5'-CCG GAA TTC ACA TTC ACG ATG TAC TTG-3'

YM003: 5'-TGC CCC CAG AGG TGC T-3'

30 The 5'-end primer ym257 of SEQ ID NO: 19 (corresponding to nucleotide Nos. 80 to 112 of SEQ ID NO: 1) was designed to replace Ser31 of HCDR1 with alanine (S31A) and the 3'-end primer YM258 of SEQ ID NO: 20 (corresponding to nucleotide Nos. 101 to 71 of SEQ ID NO: 1), to replace AGT (coding for Ser) of nucleotide Nos. 91 to 93 of HCDR1 gene with GCT (coding for alanine).

35 Each PCR reaction was carried out according to the method described in Example 1 except that primer sets, YM001N and YM258; and ym258 and YM003, were used and also that primers YM001N and YM003 were used to recombine the annealed DNA fragments obtained by PCR.

The chimeric light chain gene thus prepared was cleaved with *EcoRI* and *ApaI* and inserted at the *EcoRI/ApaI* section of vector pcDdA-chKR127HC prepared in Example 1, to obtain pcDdA-chKR127HC-S31A. The base sequence of the humanized antibody heavy chain variable region gene was confirmed by DNA sequence analysis. Vectors containing mutants thus prepared are shown in Table 1.

5 In Table 1, primer and mutation positions are numbered based on the base sequence of SEQ ID NO: 1.

Table 1

CDR	primer	primer position	mutation position	mutant	vector
HCDR1	F ym257	80-112	91-93	Ser(AGT)→ Ala(GCT)	pcDdA-chKR127HC-S31A
	R YM258	101-71			
	F ym259	83-112	94-96	Ser(TCT)→ Ala(GCT)	pcDdA-chKR127HC-S32A
	R YM260	106-73			
	F ym261	86-117	97-99	Trp(TGG)→ Ala(GCG)	pcDdA-chKR127HC-W33A
	R YM262	108-76			
	F ym263	90-118	100-102	Met(ATG)→ Ala(GCG)	pcDdA-chKR127HC-M33A
	R YM264	111-79			
	F ym265	94-120	103-105	Asn(AAC)→ Ala(GCC)	pcDdA-chKR127HC-N35A
	R ym266	112-81			
HCDR2	F YM221	139-174	148-150	Arg(CGG)→ Ala(GCC)	pcDdA-chKR127HC-R50A
	R YM222	158-128			
	F YM225	143-178	151-153	Ile(ATT)→ Ala(GCT)	pcDdA-chKR127HC-I51A
	R YM226	162-131			
	F YM227	145-180	154-156	Tyr(TAT)→ Ala(GCT)	pcDdA-chKR127HC-Y52A
	R YM228	165-135			
	F ym229	148-181	157-159	Pro(CCT)→ Ala(GCT)	pcDdA-chKR127HC-P52aA
	R YM230	167-136			
	F ym231	150-186	160-162	Gly(GGA)→ Ala(GCA)	pcDdA-chKR127HC-G53A
	R YM232	173-145			
	F ym233	152-188	163-165	Asp(GAT)→ Ala(GCT)	pcDdA-chKR127HC-D54A
	R YM234	176-144			
	F ym235	155-193	166-168	Gly(GGA)→ Ala(GCA)	pcDdA-chKR127HC-G55A
	R YM236	178-146			
	F ym237	158-194	169-171	Asp(GAT)→ Ala(GCT)	pcDdA-chKR127HC-D56A
	R ym238	184-149			
	F ym239	160-195	172-174	Thr(ACT)→ Ala(GCT)	pcDdA-chKR127HC-T57A
	R ym240	185-150			
	F ym241	164-196	175-177	Asn(AAC)→ Ala(GCC)	pcDdA-chKR127HC-N58A
	R ym242	187-150			
HCDR3	F YM207	286-317	295-297	Glu(GAG)→ Ala(GCG)	pcDdA-chKR127HC-E95A
	R YM208	305-274			
	F YM209	289-316	298-300	Tyr(TAC)→ Ala(GCC)	pcDdA-chKR127HC-Y96A
	R YM210	307-276			
	F YM211	292-318	301-303	Asp(GAC)→ Ala(GCC)	pcDdA-chKR127HC-D97A
	R YM212	313-279			
	F YM213	296-321	304-306	Glu(GAG)→ Ala(GCG)	pcDdA-chKR127HC-E98A
	R YM214	315-285			
	F YM255	303-327	310-312	Tyr(TAC)→ Ala(GGC)	pcDdA-chKR127HC-Y102A
	R YM256	319-289			

Test Example 1: Expression of chimeric antibody having a modified heavy chain and its affinity to antigen

(step 1) Expression of chimeric antibody

5 COS7 cells (ATCC CRL-1651) were seeded to DMEM media (GIBCO) containing 10% bovine serum and subcultured in an incubator at 37°C under an atmosphere of 5% CO₂. 1 x 10⁶ cells thus obtained were seeded to the same media and incubated at 37°C overnight. Thus, 5 µg of plasmid pKC-dhfr-chKR127 (expressing chimeric light chain) obtained in Example 2, 5 µg of plasmid obtained in Example 3 were diluted with OPTI-MEMI(GIBCO) to 800 µl. 50 µl of Lipofectamine (GIBCO) were diluted with the same solution to 800 µl. The resulting solutions were added to a 15 ml tube, mixed and then, kept at room temperature for more than 15 minutes. Meanwhile, COS7 cells incubated as above were washed three times with 10 OPTI-MEM I. Then, 6.4 ml of OPTI-MEM I was added to the DNA-Lipofectamine mixture and the resulting solution was evenly distributed on the COS7 cells, which were cultured for 48 hours in a 5% CO₂ incubator to obtain 15 a supernatant. The resulting solution was subjected to sandwich ELISA analysis using anti-human IgG (Sigma) as a capture antibody and anti-human 20 antigen (Fc-specific)-horseradish peroxidase (PIERCE) as a secondary antibody to confirm the expression of the chimeric antibody.

(step 2) Affinity to antigen

25 150 ng of HBV recombinant antigen GST-pre-S1(1-56) (H. S. Kim and H. J. Hong, *Biotechnology Letters*, 17, 871-876(1995)) was coated to each well of a microplate and 5 ng of the supernatant obtained in Step 1 was added to each well. The resulting solution was subjected to indirect ELISA using the same secondary antibody as used in step 1, followed by measuring the absorbance at 450 nm. Further, the affinity to antigen (K_D) of each 30 modified heavy chain was determined by competitive ELISA method (Ryu et al., *J. Med. Virol.*, 52, 226(1997)) and compared with that of pCK-dhfr-chKR127 containing wildtype chimeric heavy chain. The result is shown in Table 2.

Table 2

CDR	Mutant	K_D (nM)
	WT	11.0 \pm 1.664
H1	S31A	14.67 \pm 2.386
	S32A	8.455 \pm 0.840
	W33A	>10000
	M34A	>10000
	N35A	>10000
H2	R50A	>10000
	I51A	12.8 \pm 1.05
	Y52A	276.8 \pm 23.60
	P52aA	170.3 \pm 5.318
	G53A	7.697 \pm 0.980
	D54A	1.663 \pm 0.477
	G55A	5.766 \pm 0.211
	D56A	6.59 \pm 1.09
	T57A	13.68 \pm 4.016
H3	N58A	1.568 \pm 0.085
	E95A	>10000
	Y96A	>10000
	D97A	0.57 \pm 0.03
	E98A	64.2 \pm 7.78
	Y102A	3.581 \pm 0.457

As shown in Table 2, the affinities to antigen of the mutants obtained by replacing Trp33, Met34, or Asn35 of HCDR1; Arg50, Tyr52, or Pro52a of HCDR2; Glu95, Tyr96, or Glu98 of HCDR3 with alanine were more than 3 times lower than that of wild type. However, a mutant having alanine substituting for Asp97 or Tyr102 residue of HCDR3 exhibited an enhanced affinity to antigen.

Example 4: Preparation of HCDR3 mutants and their affinities to antigen

30 (step 1) D97R and E98V mutants

Each mutant was prepared by replacing Asp97 or Glu98 of HCDR3 with arginine as a positively charged amino acid (it is represented as "D97R") or valine as a neutral amino acid (it is represented as "E98V") according to the site-directed mutagenesis as used in Example 3. Vectors containing mutants prepared as above are shown in Table 3.

Table 3

CDR	primer		primer position	mutation position	mutant	vector
HCDR3	R	P1	312-279	301-303	Asp(GAC)→ Arg(CGG)	pcDdA-chKR127HC-D97R
	F	P2	295-326			
	R	P3	312-279	301-303	Asp(GAC)→ Val(GTT)	pcDdA-chKR127HC-D97V
	F	P4	295-326			
	R	P5	312-279	304-306	Glu(GAG)→ Arg(CGG)	pcDdA-chKR127HC-E98R
	F	P6	295-326			
	R	P7	312-279	304-306	Glu(GAG)→ Val(GTT)	pcDdA-chKR127HC-E98V
	F	P8	295-326			

Then, each mutant thus obtained was measured for its affinity to antigen in according to the method described in Test Example 1 and compared with that of the wild type.

As shown in Fig 5, the affinity to antigen of D97R was more than 3 times higher than that of the wild type, which the affinity to antigen of E98V, more than 4 times higher than that of the wild type. However, mutant E98R showed a low affinity to antigen.

(Step 2) D97R/E98V mutant

To prepare D97R/E98V mutant containing both D97R and E98V, which were found to be mutants having high affinity to antigen, PCR reaction was carried out using pcDdA-chKR127HC-D97R which contains D97R gene as a template and primers P7 and P8.

Then, the D97R/E98V mutant thus obtained was measured for its affinity to antigen in according to the method described in Test Example 1.

As shown in Fig 5, the affinity to antigen of D97R/E98V was more than 15 times higher than that of the wild type.

(Step 3) D97R/E98V/Y102A mutant

To prepare D97R/E98V/Y102A mutant containing D97R, E98V and Y102A, PCR reaction was carried out using pcDdA-chKR127HC-RV containing D97R/E98V as a template and primers YM255 and YM256.

Then, the D97R/E98V/Y102A mutant (hereinafter "RVAA") thus

obtained was measured for its affinity to antigen in according to the method described in Test Example 1.

As shown in Fig 5, the affinity to antigen of D97R/E98V/Y102A was similar to that of D97R/E98V.

5

(Step 4) D97R/E98V/Y102E and D97R/E98V/Y102R mutants

To prepare D97R/E98V/Y102E mutant and D97R/E98V/Y102R mutant, PCR reaction was carried out using pcDdA-chKR127HC-RV containing D97R/E98V as a template, and primer sets P17/P18 and P19/P20, 10 respectively.

Vector containing mutants prepared above are shown in Table 4.

Table 4

	primer		primer position	mutation position	mutant	vector
HCDR3	R	P17	312-279	307-309	Tyr(TAC)→ Glu(GAG)	pcDdA-chKR127HC-RVAE
	F	P18	295-326			
	R	P19	312-279	307-309	Tyr(TAC)→ Arg(CGT)	pcDdA-chKR127HC-RVAR
	F	P20	295-326			

Then, D97R/E98V/Y102E mutant (hereinafter "RVAE") and D97R/E98V/Y102R mutant (hereinafter "RVAR") thus obtained were measured for respective affinities to antigen in according to the method described in Test Example 1.

As shown in Fig 5, the affinity to antigen of RVAE was similar to that of RVAA, while the affinity to antigen of RVAR was higher than that of RVAA.

30 Test Example 2: Measurement of affinity to antigen of RVAR

The RVAR mutant prepared in step 4 of Example 4 was subjected to competitive ELISA to measure its affinity to antigen as follows:

35 COS7 cells were transfected with the plasmid prepared in step 4 of Example 4 and the plasmid expressing chimeric light chain(pKC-dhfr-chKR127) prepared in Example 2 to produce an antibody. 5 ng of the antibody thus obtained was reacted with pre-S1 antigen (1×10^{-7} to 1×10^{-12}

5 M) at 37 °C for 2 hours. The resulting solution was added to each well of a 96-well microplate coated with pre-S1 antigen and reacted at 37°C for 30 minutes, and then the resulting solution was subjected to ELISA analysis according to the method described in Example 4. Used as a control is chimeric antibody (chKR127) obtained from COS7 cells transfected with pcDdA-chKR127HC and pKC-dhfr-chKR127.

10 The affinity to antigen of RVAR was about 1.8×10^{-10} M, which is 45 times higher than that of chKR127, about 8.2×10^{-9} M

15 Example 5: Mutation of CDR of chimeric KR127 antibody light chain by alanine injection

20 To examine the affinity of each amino acid residue of KR127 light chain LCDR1 (aa 24-34), LCDR2(aa 50-60) and LCDR3 (aa 89-97) to antigen, PCR reaction was carried out using vector pKC-dhfr-chKR127 as a template to prepare a modified gene having each amino acid residue of CDR replaced with alanine (the replaced amino acid residue Number was indicated as Kabat number)(see Fig. 2).

25 Forward primer YM004 of SEQ ID NO: 21 was designed to provide the sequence corresponding to the reader sequence at the 5'-end of the chimeric light chain gene and the *Hind*III restriction site, and a reverse primer YM009 of SEQ ID NO: 22 was designed to have the sequence corresponding to the N-terminal region of human light chain gene and the *Bsi*WI(CGTACG) restriction site. These primers were used in preparation of mutants of light chain CDR residue.

30 YM004: 5'-CCA AAG CTT GGA AAG ATG GAT TCA CAG-3'

YM009: 5'-GCA GCC ACC GTA CGT TTG ATT TCC ACC TTG GT-3'

35 Forward primer YM135 was designed to replace Ser26 of LCDR1 with alanine (S26A) and a reverse primer YM136, to replace AGT coding for Ser at the nucleotide Nos. 76 to 78 of LCDR1 gene with GCT coding for alanine.

PCR reactions were carried out according to the method described in Example 1 except that primer sets, YM004/YM135, and YM136/YM009, were used and that primers YM004 and YM009 were used to recombine the annealed DNA fragments obtained by PCR.

The variable region gene of the mutant thus prepared was cleaved with *Hind*III and *Bsi*WI and inserted at the *Hind*III/*Bsi*WI section of vector pKC-dhfr-chKR127, to obtain pKC-dhfr-chKR127BS-S26A. The base sequence of the modified chimeric light chain variable region gene was confirmed by DNA sequence analysis. The vectors containing mutants prepared above are shown in Table 5.

In Table 5, the primer and mutation positions are numbered based on the base sequence of SEQ ID NO: 3.

10

Table 5

	primer	primer position	mutation position	mutant	vector
LCDR1	F YM135	67-102	76-78	Ser(AGT)-Ala(GCT)	pKC-dhfr-chKR127BS-S26A
	R YM136	86-54			
	F YM137	69-107	79-81	Gln(CAG)-Ala(GCG)	pKC-dhfr-chKR127BS-Q27A
	R YM138	91-56			
	F YM139	70-111	82-84	Ser(AGC)-Ala(GCC)	pKC-dhfr-chKR127BS-S27aA
	R YM140	94-58			
	F YM141	73-114	85-87	Leu(CTC)-Ala(GCC)	pKC-dhfr-chKR127BS-L27bA
	R YM142	98-64			
	F YM143	73-116	88-91	Leu(TTA)-Ala(GCA)	pKC-dhfr-chKR127BS-L27cA
	R YM144	102-68			
	F YM145	79-118	91-93	Tyr(TAT)-Ala(GCT)	pKC-dhfr-chKR127BS-Y27dA
	R YM146	103-69			
	F YM147	83-119	94-96	Ser(AGT)-Ala(GCT)	pKC-dhfr-chKR127BS-S27eA
	R YM148	107-69			
	F YM149	84-120	97-99	Asn(AAT)-Ala(GCT)	pKC-dhfr-chKR127BS-N28A
	R YM150	110-70			
	F YM151	88-127	100-102	Gly(GGA)-Ala(GCA)	pKC-dhfr-chKR127BS-G29A
	R YM152	114-74			
	F YM153	91-130	103-105	Lys(AAA)-Ala(GCA)	pKC-dhfr-chKR127BS-K30A
	R YM154	116-77			
	F YM155	93-132	106-108	Thr(ACC)-Ala(GCC)	pKC-dhfr-chKR127BS-T31A
	R YM156	118-80			
	F YM103	99-133	109-111	Tyr(TAT)-Ala(GCT)	pKC-dhfr-chKR127BS-Y32A
	R YM104	120-83			
	F N34A-F	106-132	115-118	Asn (AAT)-Ala(GCT)	pKC-dhfr-chKR127BS-Y34A
	R N34A-R	126-100			

		primer	primer position	mutation position	mutant	vector
5	LCDR2	F YM129	151-188	163-165	Leu(CTG)-Ala(GCG)	pKC-dhfr-chKR127BS-L50A
		R YM130	175-140		Val(GTG)-Ala(GCG)	pKC-dhfr-chKR127BS-V51A
		F YM131	153-191	166-168	Ser(TCT)-Ala(GCT)	pKC-dhfr-chKR127BS-S52A
		R YM132	179-145		Lys(AAA)-Ala(GCA)	pKC-dhfr-chKR127BS-K53A
		F YM133	157-192	169-171	Leu(CTG)-Ala(GCG)	pKC-dhfr-chKR127BS-L54A
		R YM134	181-147		Asp(GAC)-Ala(GCC)	pKC-dhfr-chKR127BS-D55A
		F K53A-F	163-187	172-174	Ser(TCT)-Ala(GCT)	pKC-dhfr-chKR127BS-S56A
		R K53A-R	178-154		Val(GTG)-Ala(GCA)	pKC-dhfr-chKR127BS-V89A
		F L54A-F	163-189	175-177	Gln(CAA)-Ala(GCA)	pKC-dhfr-chKR127BS-Q90A
		R L54A-R	180-159		Gly(GGT)-Ala(GCT)	pKC-dhfr-chKR127BS-G91A
10	LCDR3	F D55A-F	170-195	178-180	Thr(ACA)-Ala(GCA)	pKC-dhfr-chKR127BS-T92A
		R D55A-R	184-163		His(CAT)-Ala(GCT)	pKC-dhfr-chKR127BS-H93A
		F K56A-F	175-198	181-183	Phe(TTT)-Ala(GCT)	pKC-dhfr-chKR127BS-F94A
		R K56A-R	190-168		Pro(CCT)-Ala(GCT)	pKC-dhfr-chKR127BS-P95A
		F YM113	270-304	280-282	Gln(CAG)-Ala(GCG)	pKC-dhfr-chKR127BS-Q96A
		R YM114	292-258		Thr(ACG)-Ala(GCG)	pKC-dhfr-chKR127BS-T97A
		F YM115	274-307	283-285	Val(GTG)-Ala(GCG)	pKC-dhfr-chKR127BS-V89A
		R YM116	294-259		Gln(CAA)-Ala(GCA)	pKC-dhfr-chKR127BS-Q90A
		F YM117	277-310	286-288	Gly(GGT)-Ala(GCT)	pKC-dhfr-chKR127BS-G91A
		R YM118	296-265		Thr(ACA)-Ala(GCA)	pKC-dhfr-chKR127BS-T92A
15	LCDR3	F YM119	281-310	289-291	His(CAT)-Ala(GCT)	pKC-dhfr-chKR127BS-H93A
		R YM120	302-266		Phe(TTT)-Ala(GCT)	pKC-dhfr-chKR127BS-F94A
		F YM121	282-313	295-297	Pro(CCT)-Ala(GCT)	pKC-dhfr-chKR127BS-P95A
		R YM122	304-271		Gln(CAG)-Ala(GCG)	pKC-dhfr-chKR127BS-Q96A
		F YM123	286-317	301-303	Thr(ACG)-Ala(GCG)	pKC-dhfr-chKR127BS-T97A
		R YM124	308-278		Val(GTG)-Ala(GCG)	pKC-dhfr-chKR127BS-V89A
		F YM125	292-319	304-306	Gln(CAA)-Ala(GCA)	pKC-dhfr-chKR127BS-Q90A
		R YM126	311-279		Gly(GGT)-Ala(GCT)	pKC-dhfr-chKR127BS-G91A
		F YM127	294-320	304-306	Thr(ACA)-Ala(GCA)	pKC-dhfr-chKR127BS-T92A
		R YM128	313-282		His(CAT)-Ala(GCT)	pKC-dhfr-chKR127BS-H93A

30

Test Example 3: Measurement of affinity to antigen of light chain mutant

35 COS7 cell was transfected with each of the light chain mutants prepared in Example 5 and the plasmid expressing chimeric heavy chain(pcDdA-chKR127HC) to produce an antibody. The antibody obtained

was measured for its affinity to antigen in accordance with the method described in Test Example 1.

Table 6 shows the results obtained for the mutants and pdDA-chKR127HC containing wildtype chimeric KR127 heavy chain.

5

Table 6

	CDR	mutant	K _D (nM)
10	L1	S26A	6.49 ± 0.244
		Q27A	14.2 ± 2.29
		S27aA	37.9 ± 6.66
		L27bA	>10000
		L27cA	36.8 ± 11.01
		Y27dA	1032.7 ± 56.1
		S27eA	>10000
		N28A	>10000
		G29A	23.94 ± 2.62
		K30A	>10000
		T31A	13.19 ± 1.98
		Y32A	>10000
		N34A	>10000
15	L2	L50A	159.4 ± 21.37
		V51A	37.00 ± 10.33
		S52A	14.08 ± 0.509
		K53A	7.928 ± 0.976
		L54A	12.57 ± 2.453
		D55A	225.2 ± 2.970
		S56A	12.95 ± 0.367
20	L3	V89A	121.2 ± 4.62
		Q90A	>10000
		G91A	>10000
		T92A	74.2 ± 2.90
		H93A	54.5 ± 4.48
		F94A	>10000
		P95A	>10000
		Q96A	293.6 ± 7.13
		T97A	17.3 ± 2.56
25			

30 As shown in Table 6, the affinities to antigen of the mutants obtained by replacing the Leu27b, Tyr27d, Ser27e, Asn28, Lys30, Tyr32, and Asn34 of LCDR1; Leu50 and Asp55 of LCDR2; and Val89, Gln90, Gly91, Thr92, His93, Phe94, Pro95, and Gln96 of LCDR3 with alanine, respectively, were more than 3 times lower than that of the wild type. Therefore, these residues was determined as SDR.

35

Example 6: Preparation of humanized heavy chain by SDR-grafting method

A humanized heavy chain was prepared using DP7-JH4, a human heavy chain constructed by combining human immunoglobulin germline VH gene segment DP7 (Tomlinson et al., *J. Mol. Biol.*, 227, 776-798, 1992) having an amino acid sequence similar to KR127 heavy chain variable regions and human immunoglobulin germline JH4 segment (Ravetch et al., *Cell*, 27, 583-591 (1981)).

5 The Trp33 and Asn35 in HCDR1 of the KR127 were grafted into the DP7-JH4. The Met34 in HCDR1 of the KR127 is identical to that of DP7-JH4. Further, to inhibit lowering the affinity to antigen, Tyr32 in HCDR1 10 of the KR127 was replaced with alanine of HCDR1 of a human antibody (Gen Bank data base 75023 (SAWMN)).

15 The Arg50 and Tyr52 in HCDR2 of the KR127 were grafted onto the DP7-JH4. The Pro52a in HCDR2 of the KR127 is identical to that of DP7-JH4.

15 The Asp95, Tyr96, Arg97, Val98, and Arg102 of HCDR3 were grafted into DP7-JH4.

20 Further, Ala71 and Lys73 of FR 3 (framwork region 3) in the heavy chain variable region of KR127 antibody which affects the conformation of CDR loops were grafted thereto.

25 Then, PCR reaction was carried out using primers Ryu166 of SEQ ID NO: 23 and Hur37 of SEQ ID NO: 24 according to the method described in Example 3 to obtain a humanized heavy chain variable region gene, HuKR127VH-VII.

25 Ryu 166: 5'-GGA TTT GTC TGC AGT CAT TGT GGC TCT GCC CTG
GAA CTT-3'

Hur 37: 5'-GAC AAA TCC ACG AGC ACA GTC TAC ATG-3'

30 The base sequence of the humanized heavy chain variable region gene was determined by DNA sequence analysis (Fig. 2). Then, the gene was cleaved with *EcoRI* and *Apal* and inserted at the *EcoRI/Apal* section of vector pdDdA-chKR127HC to obtain pHuKR127HC.

35 A humanized antibody was prepared by combining humanized heavy chain thus obtained and the humanized antibody HZKR127I light chain described in Korean Patent No. 246128 and measured the affinity to antigen

was numbered according to the method described in Test Example 2. Humanized antibody HZKR127I was used as a control.

The affinity to antigen of the humanized antibody of about 1.5×10^{-10} M was about 50 times higher than that of HZKR127I, about 8.2×10^{-9} M.

5

Example 7: Preparation of humanized light chain by SDR-grafting method

A humanized light chain was prepared using DP7-JH4, a human light chain constructed by combining human immunoglobulin germline VK gene segment DPK12 (Cox et al., *Eur. J. Immunol.*, 24, 827-836 (1994)) having an amino acid sequence similar to KR127 light chain variable regions and human immunoglobulin germline JK4 segment (Hieter et al., *J. Biol. Chem.*, 257, 1516-1522 (1982)).

The Tyr27d, Asn28 and Asn34 in LCDR1 of KR127 were grafted into the DPK12-JK4. The amino acid residues at position 27b, 27e, 30 and 15 32 of DP7 is identical to those of KR127 light chain.

The Leu50 and Asp55 in LCDR2 of KR127 were grafted into the DPK12-JK4 gene.

The Val89, Gly91, Thr92, His93, Phe94, and Gln96 in LCDR3 of KR127 were grafted into the DPK12-JK4. The residues at positions 90 and 20 95 of DP7 is identical to those of KR127.

Further, Leu36 and Arg46 of FR 2 in the light chain variable region of KR127 antibody (which acts on interaction with heavy chain or CDR) were grafted thereto.

Then, PCR reaction was carried out using primers Ryu118 of SEQ ID NO: 25 and Ryu119 of SEQ ID NO: 26 according to the method described in Example 3 to prepare a humanized light chain variable region gene, HuKR127VH-IV.

Ryu 118: 5'-CTG TGG AGG CTG GCC TGG CTT CTG TAA TAA CCA-3'

30 Ryu 119: 5'-GGC CAG CCT CCA CAG CTC CTA ATC TAT CTG-3'

The base sequence of the humanized light chain variable region gene was determined by DNA sequence analysis (see HZIV of Fig. 4). Then, the gene was cleaved with *Hind*III and *Bsi*WI and inserted at the *Hind*III/*Bsi*WI section of vector pKC-dhfr-chKR127BS to obtain pHuKR127KC.

35 A humanized antibody was prepared by combining humanized light

chain thus obtained and the humanized antibody HZKR127I heavy chain described in Korean Patent No. 246128 and its affinity to antigen was measured according to the method described in Test Example 2. Humanized antibody HZKR127I was used as a control.

5 The affinities to antigen of the humanized antibody of about 8.4×10^{-9} M was similar to that of HZKR127I, about 8.2×10^{-9} M.

Example 8: Preparation of humanized antibody and measurement of the affinity to antigen

10 To prepare a plasmid containing humanized heavy chain plasmid pHuKR127HC and humanized light chain plasmid pHuKR127KC, the *EcoRI/ApaI* fragment containing humanized heavy chain variable region gene of pHuKR127HC and the *HindIII/BsiWI* fragment containing humanized light chain variable region gene of pHuKR127KC were inserted 15 at the *EcoRI/ApaI* and *HindI/BsiWI* sections of vector pdCMV-dhfrC-HAV6 (KCTC 10028BP), respectively, to obtain plasmid pdCMV-dhfrC-HuKR127 (Fig. 6). *E. coli* DH5 α was transformed with the plasmid thus obtained and the transformed *E. coli* DH5 α /pdCMC-dhfrC-HuKR127 was deposited 20 on March 13, 2002 with the Korean Collection for Type Cultures(KCTC)(Address: Korea Research Institute of Bioscience and Biotechnology(KRIBB), #52, Oun-dong, Yusong-ku, Taejon, 305-333, Republic of Korea) under the accession number, KCTC 10198BP, in accordance with the terms of Budapest Treaty on the International 25 Recognition of the Deposit of Microorganism for the Purpose of Patent Procedure.

To prepare cell line expressing the humanized antibody, dhfr-defected CHO (chinese hamster ovary) cells were transformed with plasmid pdCMV-dhfrC-HuKR127 as follows:

CHO cells (ATCC CRL 9096) were seeded to DMEM/F12 media 30 (GIBCO) containing 10% fetal bovine serum and subcultured in an incubator at 37°C under an atmosphere of 5% CO₂. 5×10^5 cells thus obtained were seeded to the same media and incubated at 37°C overnight, followed by washing 3 times with OPTI-MEMI solution (GIBCO).

Meanwhile, 5 μ g of the plasmid pdCMV-dhfrC-HuKR127 was 35 diluted in 500 μ l of OPTI-MEMI solution. 25 μ l of Lipofectamine was diluted in 500 μ l of the same solution. The resulting solutions were added

to a 15 ml tube, mixed, and then, kept at room temperature for more than 15 minutes. Then, 2 ml of OPTI-MEM I was added to by DNA-Lipofectamine mixture and the resulting solution was distributed evenly on the COS7 cells to be kept in a 5% CO₂ incubator at 37°C for 6 hours. Added thereto was 3 ml of DMEM/F12 containing 20% fetal bovine serum and cultured for 48 hours.

Then, CHO cells were taken up with trypsin and cultured in a-MEM media(GIBCO) of 10 % dialyzed fetal bovine serum containing G418 (GIBCO BRL, 550 mg/l) for 2 weeks. After confirming of antibody-producing ability of the transformed clone, the clone was cultured in a-MEM media of 10 % dialyzed fetal bovine serum containing 20nM MTX to induce amplification of gene.

Cell line CHO/HuKR127 having the highest antibody-productivity was selected from the clones and deposited on March 13, 2002 with the Korean Collection for Type Cultures(KCTC) under the accession number, KCTC 10199BP, in accordance with the terms of Budapest Treaty on the International Recognition of the Deposit of Microorganism for the Purpose of Patent Procedure.

To measure the affinity to antigen of the humanized antibody HuKR127, CHO cell line thus obtained was mass cultured in a serum-absence media (CHO-SFMII, GIBCO) and subjected to protein G-shepharose 4B column (Pharmacia). Then, the antibody absorbed on the column was eluted with 0.1 M glycine solution (pH 2.7) and neutralized with 1.0 M tris solution (pH 9.0), followed by dialyzing in PBS buffer (pH 7.0). Further, the affinity to antigen of the purified antibody was determined by the competitive ELISA method described in Test Example 2 and compared with that of a control, humanized HuKR127I. The result was shown in Fig. 7.

As shown in Fig. 7, the affinity to antigen of the humanized antibody of the present invention of 1.6×10^{-10} M was about 50 times higher than 8.2×10^{-9} M of the control group.

Example 9: Confirmation of immune-response induction of humanized antibody

To confirm whether the humanized antibody of the present invention (HuKR127) prevents HAMA response, an analysis was conducted according to the TEPITOPE method (Sturniolo et al., *Nature Biotechnology*, 17, 555-

561, 1999) to examine whether a peptide sequences which can bind to MHC (major histocompatibility complex) class II exists in the heavy and light chain variable regions of the humanized antibody.

5 Tables. 7 and 8 show the results of such analysis for MHC class II-binding peptide sequences in the heavy chain variable regions of HuKR127 and the light chain variable regions of HuKR127, respectively.

Table 7

antibody	HzKR127I		HuKR127	
	peptide	MHC class II	peptide	MHC class II
MHC class II -binding	LVQSGAEVV	DRB1_0306 DRB1_0307 DRB1_0308 DRB1_0311 DRB1_0421 DRB1_0701 DRB1_0703	LVQSGAEVK	0
	VKPGASVVK	DRB1_0102	KKPGASVVK	0
	FSSSWMNIVV	DRB1_0703	FTSAWMNIVV	0
	WIGRIYPGD	DRB1_0801 DRB1_0817	WMGRIYPSG	0
	FQGKATLTA	DRB1_0401 DRB1_0402 DRB1_0405 DRB1_0408 DRB1_0421 DRB1_0426 DRB1_0801 DRB1_0802 DRB1_0804 DRB1_0806 DRB1_0813 DRB1_0817 DRB1_1101 DRB1_1102 DRB1_1104 DRB1_1106 DRB1_1114 DRB1_1120 DRB1_1121 DRB1_1128 DRB1_1302 DRB1_1305 DRB1_1307 DRB1_1311 DRB1_1321 DRB1_1322 DRB1_1323	FQGRVTMTA	DRB1_0305 DRB1_0401 DRB1_0402 DRB1_0408 DRB1_0426 DRB1_0801 DRB1_0802 DRB1_0804 DRB1_0806 DRB1_0813 DRB1_0817 DRB1_1101 DRB1_1114 DRB1_1120 DRB1_1128 DRB1_1302 DRB1_1305 DRB1_1307 DRB1_1321 DRB1_1323 DRB1_1502
	YWGQGTIVT	DRB1_0401 DRB1_0405 DRB1_0421 DRB1_0426	RWGQGTIVT	0
	IGRIYPGDG	DRB5_0101 DRB5_0105	MGR1YPSGG	DRB1_0404 DRB1_0405 DRB1_0410 DRB1_0423
	YAQKFQGKA	DRB1_0802	YAQKFQGRV	0
	VYFCAREYD	DRB1_1304	VYYCAREYR	DRB1_0301
	YWGQGTIVT	DRB1_0401 DRB1_0405 DRB1_0421 DRB1_0426	RWGQGTIVT	0
total		50		26

Table 8a

antibody	HzkR127I		HuKR127	
	peptide	MHC class II	peptide	MHC class II
MHC class II -binding	ILMTQTPLS	DRB1_0301 DRB1_0305 DRB1_0306 DRB1_0307 DRB1_0308 DRB1_0309 DRB1_0311 DRB1_0401 DRB1_0402 DRB1_0404 DRB1_0405 DRB1_0408 DRB1_0410 DRB1_0421 DRB1_0423 DRB1_0426 DRB1_0804 DRB1_1101 DRB1_1102 DRB1_1104 DRB1_1106 DRB1_1107 DRB1_1114 DRB1_1121 DRB1_1128 DRB1_1301 DRB1_1304 DRB1_1305 DRB1_1307 DRB1_1311 DRB1_1321 DRB1_1322 DRB1_1323 DRB1_1327 DRB1_1328	IVMTQTPLS	0
		DRB1_0101 DRB1_0102 DRB1_1304		
	WLLQKPGQS	DRB1_0101 DRB1_0305 DRB1_0309 DRB1_0401 DRB1_0408 DRB1_0421 DRB1_0426 DRB1_0802 DRB1_1101 DRB1_1107 DRB1_1114 DRB1_1120 DRB1_1128 DRB1_1302 DRB1_1305 DRB1_1307 DRB1_1321 DRB1_1323 DRB5_0101 DRB1_0105	WLLQKPGQP	0
		DRB1_0101 DRB1_0701 DRB1_0703 DRB5_0101 DRB5_0105		
	YCVQGTHF	DRB1_0401 DRB1_0421 DRB1_0426	YCVQGTHFP	DRB1_0401 DRB1_0421 DRB1_0426

Table 8b

antibody	HzKR127I		HuKR127		
	peptide	MHC class II	peptide	MHC class II	
	VGVYYCVQG	DRB1_0806	VGVYYCVQG	DRB1_0806	
5	IYLVSKLDS	DRB1_0301			
		DRB1_0305			
		DRB1_0306			
		DRB1_0307			
		DRB1_0308			
		DRB1_0309			
		DRB1_0311			
		DRB1_0405			
		DRB1_0410			
		DRB1_0801			
		DRB1_0802			
		DRB1_0804			
		DRB1_0806			
		DRB1_0813			
		DRB1_0817			
		DRB1_1101			
		DRB1_1102			
		DRB1_1104			
		DRB1_1106			
		DRB1_1107			
		DRB1_1114			
		DRB1_1120			
		DRB1_1121			
		DRB1_1128			
		DRB1_1301			
		DRB1_1302			
		DRB1_1304			
		DRB1_1305			
		DRB1_1307			
10		DRB1_1311			
		DRB1_1321			
		DRB1_1322			
		DRB1_1323			
		DRB1_1327			
		DRB1_1328			
15	IYLVSNRDS	DRB1_1501			
		DRB1_1506			
				DRB1_0402	
				DRB1_0404	
				DRB1_0405	
20	LIYLVSKLD	DRB1_0806		DRB1_0408	
		DRB1_1304		DRB1_0410	
		DRB1_1321		DRB1_1311	
				DRB1_1322	
				DRB1_1323	
				DRB1_1327	
				DRB1_1328	
				DRB5_0101	
				DRB5_0105	
25	LIYLVSNRD			DRB1_0401	
				DRB1_0404	
				DRB1_0405	
				DRB1_0408	
30	YLVSKLDSG	DRB1_0410		DRB1_0410	
		DRB1_0421		DRB1_0421	
		DRB1_0423		DRB1_0423	
35	YLVSNRDSG	DRB1_0426		DRB1_0426	
		DRB1_1304		DRB1_1304	
total		106		40	

As can be seen from Figs. 7 and 8, the number of the peptide sequence in the humanized antibody HuKR127 which binds to MHC class II was fewer than of that the HzKR127I. These results suggest that humanized antibody HuKR127 of the present invention is expected to reduce HAMA response to a greater extent than HzKR127I.

While the embodiments of the subject invention have been described and illustrated, it is obvious that various changes and modifications can be made therein without departing from the spirit of the present invention which should be limited only by the scope of the appended claims.

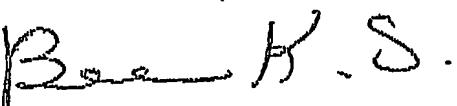
BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT
OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : HONG, Hyo Jeong
 Clover Apt. 117-201, Dunsan-dong, Seo-ku, Taejon 302-772,
 Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: <i>Escherichia coli</i> DH5@/pdCMV-dhfrC-HuKR127	Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY: KCTC 10198BP
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
<p>The microorganism identified under I above was accompanied by:</p> <p><input checked="" type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)</p>	
III. RECEIPT AND ACCEPTANCE	
<p>This International Depositary Authority accepts the microorganism identified under I above, which was received by it on March 13 2002.</p>	
IV. RECEIPT OF REQUEST FOR CONVERSION	
<p>The microorganism identified under I above was received by this International Depositary Authority on _____ and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on _____</p>	
V. INTERNATIONAL DEPOSITORY AUTHORITY	
Name: Korean Collection for Type Cultures Address: Korea Research Institute of Bioscience and Biotechnology (KRIIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or authorized official(s):  BAE, Kyung Seok, Director Date: March 16 2002

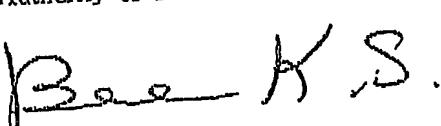
BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT
OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : HONG, Hyo Jeong
 Clover Apt. 117-201, Dunsan-dong, Seo-ku, Taejon 302-772,
 Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: CHO/HinKR127 (CHO cell line)	Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY: KCTC 10199BP
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
<p>The microorganism identified under I above was accompanied by:</p> <p><input checked="" type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)</p>	
III. RECEIPT AND ACCEPTANCE	
<p>This International Depositary Authority accepts the microorganism identified under I above, which was received by it on March 13 2002.</p>	
IV. RECEIPT OF REQUEST FOR CONVERSION	
<p>The microorganism identified under I above was received by this International Depositary Authority on _____ and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on _____</p>	
V. INTERNATIONAL DEPOSITORY AUTHORITY	
Name: Korean Collection for Type Cultures Address: Korea Research Institute of Bioscience and Biotechnology (KRIIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or authorized official(s):  BAE, Kyung Sook, Director Date: March 16 2002